PREFERENTIAL METHYLATION OF REGULATORY GENES IN HELA CELLS

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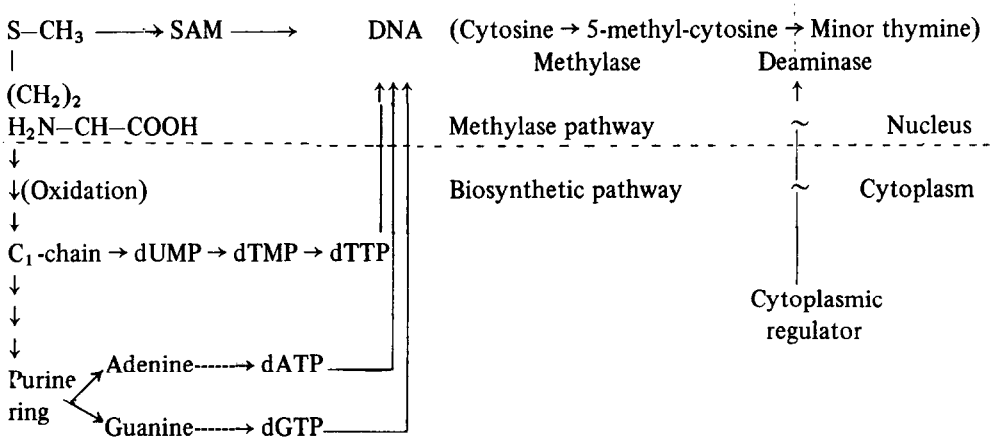
1. Introduction

The genome of a cell is exposed to the action of two classes of DNA-modifying enzymes: (1) a DNA methylase which transforms roughly 1/15 of its cytosines to 5-methyl-cytosines, while (2) a mere 1/1000 of these 5-methyl-cytosines are transformed by a DNA deaminase to minor thymines [1–3]. Transformation from cytosine to 5-methylcytosine can take place in isolated nuclei of sea urchin eggs [4] and HeLa cells [5]. But, at least for HeLa cells, transformation from 5-methylcytosine to 'minor' thymine does not take place in isolated nuclei [5] and probably is under cytoplasmic control. This was suggested by investigation on synchronized HeLa cell suspension in which [^{14}C]methyl-L-methionine was employed as the sole tracer for both methylation and synthesis of DNA [5]:

The labelled C-atom of the methyl group of L-methionine does not enter the pyrimidine ring [2]: *via methylation* it is transferred to DNA 5-methylcytosine; *via C_1-intermediates* it enters the purine ring and the methyl group of thymine to participate in the DNA biosynthetic process. The two pathways of DNA methylation and DNA synthesis were then separated from each other during the HeLa cell cycle [5]: in the whole cell, DNA methylation parallels DNA synthesis during the S-phase; in the isolated nuclei, DNA methylation proceeds during the S-phase in the absence of DNA synthesis. Therefore, completed DNA chains can be methylated (furthermore, Adams [6] has demonstrated that newly synthesized chains are not methylated); their methylation during S might represent a condition of DNA duplication or of gene expression [2,4].



Several considerations led to concentrating efforts on investigating a possible role of DNA methylation in the control of transcription. Methylation, in fact, does not occur at random on DNA. The GC→AT transitions [2,4] are unlikely to be meaningless. In the sea urchin, methylation involves preferentially the C monopyrimidine isostichs and the GC-dinucleotides [2]. In mammals, cytosines are preferentially methylated when in the sequence CpG [7]. Thus, if the regulatory zone of Georgiev's operon in eukaryotes is GC-enriched in comparison with the structural zone [8], one would expect it to be exposed more to methylation.

The results presented here suggest that in HeLa cells the regulatory genes indeed appear to be more involved in methylation. The following discussion concerns the biochemical differentation and the probable site of attachment of virus genome to the host cell genome.

2. Materials and methods

2.1. *Cells and synchronization*

HeLa S3 cells were grown in suspension [9] and synchronized with a double thymidine block as described earlier [10].

2.2. *DNA*

A synchronized suspension, containing 500×10^6 cells in one 1, was allowed to reach mid S-phase. Nuclei were prepared according to Penman [11], washed in 0.1 M Tris–HCl, pH 7.4, and then incubated as described elsewhere [5] in 2 ml of 0.003 M Tris–HCl, pH 7.4, for 30 min at 37°C with 500 μCi of [^{3}H]+S-Adenosyl-L-methionine (8.02 Ci/m mole). DNA, in which only 5-methylcytosine was labelled [5], was extracted and purified with minor modification of Marmur's method [12]. Extensive shearing by sonication to fragments of about $6 \times 10^4 - 10^5$ daltons and denaturation were performed according to Shenkin and Burdon [13].

2.3. *dRNA and mRNA*

To label dRNA, 100×10^6 cells synchronized in mid S-phase were suspended in 100 ml of Joklic-modified minimum essential medium containing 10% calf serum, 0.04 μg/ml Actinomycin D [14] and 500 μCi of [^{14}C]uridine (56.7 mCi/mmole). Incubation lasted 30 min at 37°C with magnetic stirring under a continuous flow of 5% CO_2 in air. Incorporation of radioactivity was stopped by chilling. Nuclei were separated [11] and the dRNA was prepared as in [15–17]. Under these conditions the 45 S rRNA precursor was practically unlabelled, while the rate of labelling of dRNA was still linear [14]. Radioactive dRNA analysed on 15–30 sucrose-gradients sedimented in the range 10–80 S [18].

To prepare mRNA, 1×10^9 cells synchronized in mid S-phase were suspended in 1 l of Joklic-modified minimum essential medium supplemented with 10% calf serum, containing 0.04 μg/ml Actinomycin D [14] and 2 mCi of [^{14}C]uridine (56.7 mCi/mmole). Labelling lasted 30 min at 37°C with magnetic stirring under aeration with 5% CO_2 and terminated by chilling. Polysomes were extracted from the cytoplasmic fraction after sedimenting the nuclei and purified on 7–47% sucrose gradients in reticulocyte standard buffer (0.01 M NaCl; 0.0015 M $MgCl_2$; 0.01 M Tris–HCl, pH 7.4). Gradients did not reveal appreciable radioactivity on ribosomal subunits or on the monomer, whereas all counts were associated with polysomes. Therefore, the 28 and 18 S rRNA were practically unlabelled. The gradient fractions containing polysomes were pooled and appropriately treated with hot-phenol [15–17] to release mRNA. Radioactive mRNA analysed on 15–30% sucrose gradients sedimented in the range 10–30 S [18].

2.4. *DNA/RNA hybridization*

dRNA and mRNA were hybridyzed with DNA in solution, since the liquid system was found quite practical for analysis of the DNA fractions recovered from CsCl gradients [13]. Hybrids were purified easily as in [13,19,20]. Because of the C_0t disproportion in hybridizing DNA with dRNA and mRNA [21], the time of hybridization of DNA with mRNA was much longer than that with dRNA.

3. Results

3.1. *DNA methylation in early and late S-phase*

Evidence existed showing that S-phase is subdivided into two parts with respect to the characteristics of the DNA replicons: in several cell species, early replicating

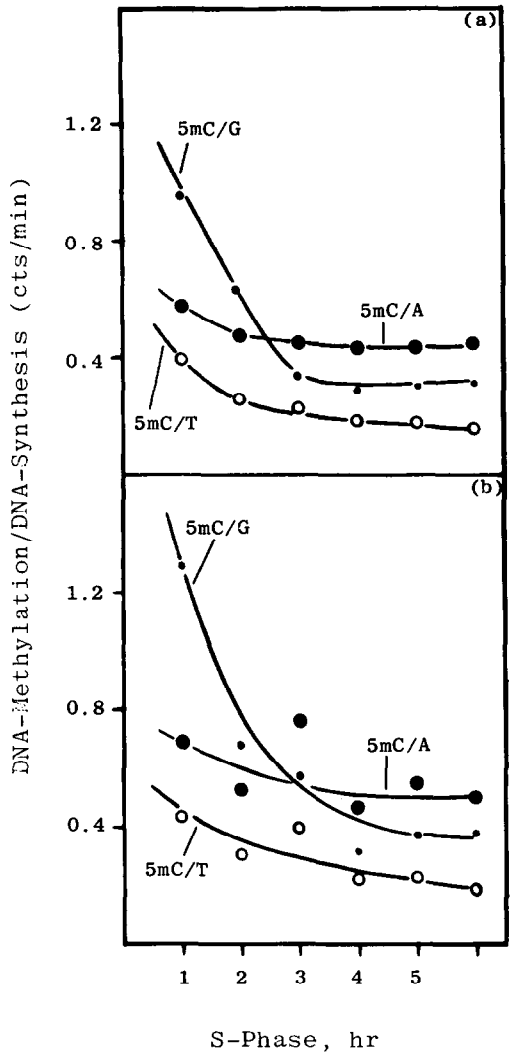


Fig. 1. Specific DNA methylation during the S-phase of HeLa cells. Curves are drawn from data described earlier [5]. Abscissa, time of removal of synchronizing thymidine from the suspension culture [10]. Ordinate, ratios of radioactivities relating to DNA methylation, 10^3 cpm 5-methyl-cytosine (5 mC), and to DNA synthesis, 10^3 cpm guanine (G), adenine (A) and thymine (T). (a) Whole cells: [^{14}C]methyl-L-methionine was the sole tracer for both DNA methylation and DNA synthesis [5]. (b) Isolated nuclei: DNA-5-methyl-cytosine was labelled with [^{3}H]S-adenosyl-L-methionine in the absence of DNA synthesis; the radioactivities of DNA guanine (G), adenine (A) and thymine (T) are the same as in (a).

euchromatic DNA tends to be GC-rich, while late replicating heterochromatic DNA tends to be AT-rich [22,23]. In Chinese hamster cells, DNA extracted during the early S-phase is methylated to a greater degree than that extracted during the late S-phase [23]. Fig. 1 shows this also to be true of HeLa cells, and in addition shows that methylation of DNA in the early S-phase is much higher than expected from the GC content of the already synthetized DNA, since the 5 mCG curve during the first part of the S-phase is strikingly steep if compared with the 5 mCA and 5 mCT curves. Thus, the degree of methylation of DNA seems to depend not simply on the proportion of CG pairs, but C in the euchromatic fraction of DNA synthetized in early S-phase is more methylated than in the heterochromatic AT-rich DNA synthetised in late S.

3.2. *Hybrids of methylated DNA with dRNA and mRNA*

Some information tends to restrict further the localization of methylation on DNA. Although the dA-rich and dG-rich regions have a wide spread distribution throughout DNA molecules [13], it is suggested that pyrimidine-rich clusters might serve as sites for binding RNA polymerase [24]. But C pyrimidine isostichs, as mentioned, were found to be highly methylated [2]. Moreover, arginine-rich histones have a greater affinity for GC-rich regions of DNA [25], and data showed a correlation between the distribution of fl histones on DNA and the occurrence of repetitive sequences [26]. But these, with the exception for rRNA genes, are known to amplify – as a general rule – the information of the regulatory region of the operon in eukaryotes [8,26]. On the other hand, it was found that addition of small amounts of trypsin to isolated nuclei of sea urchin eggs [4] produces a 20-fold increase in DNA methylation. Trypsin is specific for basic amino acid chains. Thus, the arginine-rich histones, bound to GC-regions of DNA [25,26], might be masking the sites for DNA methylation. Finally, according to the model of the operon in eukaryotes, the 3′-end of dRNA must be AT-rich and GC-poor [8]. It remained, thus, to verify with a more direct experiment whether the sequences proximal to promotor (5′-end) are preferentially methylated.

Fig. 2a confirms for HeLa cells the information

obtained with Chinese hamster cells [23] on supermethylation of GC-rich DNA pieces sedimenting in the denser region of the CsCl gradient. Therefore, it strongly supports the data of fig. 1. Furthermore, when the methylated DNA is hybridized with the nuclear giant dRNA molecule, which is the first product of transcription complementary to the whole operon [8,26], an appreciable amount of ^3H counts, signifying DNA methylation, is found in the hybrids (fig. 2b). The result is not surprising, because dRNA is supposed to bind the operon from the 5′ to the 3′-end [8,26]. When, instead, the same methylated DNA is hybridized with polysomal mRNA, which after the cleavage in nuclei of the dRNA precursor molecule brings to the cytoplasm the message complementary only to the structural region of the operon [8,26], practically no significant ^3H counts are found in the hybrids (fig. 2c). Thus, the DNA region containing methylated bases was left out by mRNA. This region is supposed to be proximal to the promotor 'acceptor' zone of the operon [8].

4. Discussion

The results presented here should be considered with some caution because of the complexity of the cellular processes involved. Their interpretation is based, however, on some clear points: (a) polysomal mRNA is a product of dRNA processing [8,18]; (b) it represents the segment of dRNA which is complementary to the structural zone of the operon [8,18,26]; (c) the site of attachment of RNA polymerase (promotor) appears to be a poly-C sequence

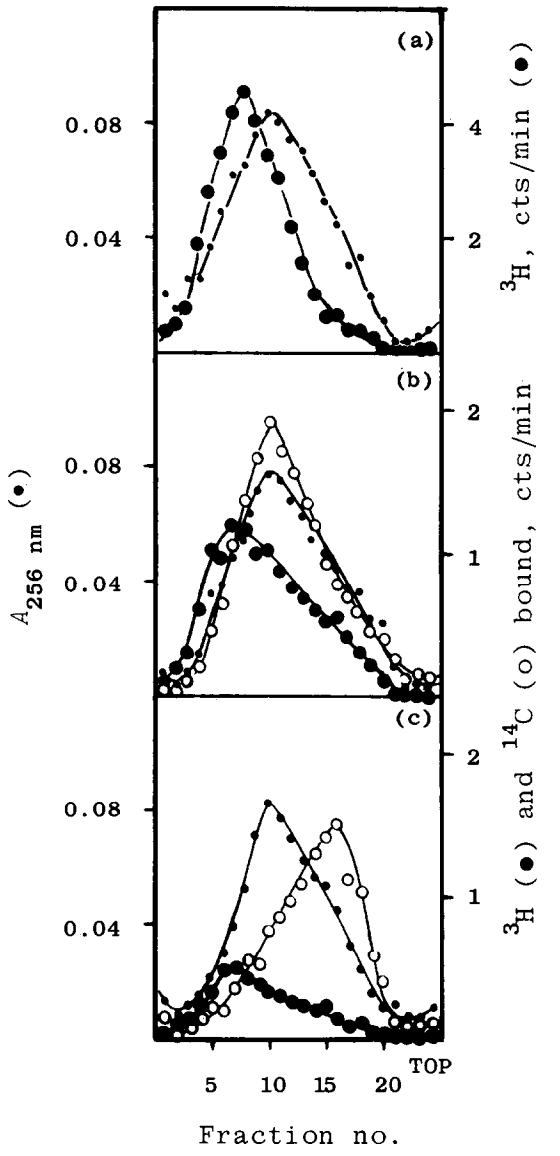


Fig. 2. Equilibrium ultracentrifugation of methylated DNA and hybridization of the density gradient fractions with dRNA and mRNA. 75 μg of sheared [^{3}H]DNA in 0.5 ml of 0.1 × SSC (1× = 0.15 M NaCl, 0.015 M sodium citrate) were added to 4.5 ml of 1.78 g cm^{-3} CsCl solution and the final density was adjusted to 1.700 g cm^{-3}. The samples were run at 33 000 rpm at 20°C in a Spinco 39 rotor for 64 hr. Fractions (5 drops) were collected into 0.5 ml of 0.1 SSC and analyzed. (a) After measurement of absorbance [12,35], the collected fractions were directly precipitated with 10% trichloroacetic acid (vol/vol) and tested for radioactivity cpm × 10^4). (b) After measurement of absorbance, fractions were dialysed against 2 changes of 10 ml of 2 × SSC. To each of them 0.5 ml of dRNA (in 2 × SSC) with 52 000 cpm was added and the final volume was adjusted to 2 ml of 2 × SSC. Mixtures were boiled for 15 min and rapidly cooled. Further incubation lasted 2 hr at 62°C. Digestion with 10 μg/ml RNAse was performed at room temperature for 30 min. The purified [13,19,20] hybridized material was precipitated with 5% trichloroacetic acid and tested for both ^{3}H and ^{14}C cpm (× 10^2). (c) Procedure was as in (b) except that the gradient fractions were mixed with 0.5 ml of mRNA (in 2 × SSC) with 37 000 cpm, and the incubation lasted 24 hr at 62°C.

[24], while the sequences complementary to polysomal mRNA seem to be AT-rich [8], and (d) the messenger ribonucleoprotein complex, distributed in the region 6–28 S with a main peak at 15 S, has a high AU/GC ratio of 1.18 [27]. On the other hand, the correlation between the GC-richness and supermethylation in DNA is largely documented (figs. 1 and 2a, and ref. [23]); methylation of DNA is increased by trypsin which dissociates Arg-rich histones [4]; the fl histone is specifically bound to the regulatory zone of Georgiev's operon [26]. Therefore, the undermethylation of the structural genes, suggested by the results presented in fig. 2c, appears not to be in contradiction with the prediction that structural genes should not undergo C → 5 mC → T mutations in order to bring about the synthesis of normal proteins in the cell. A preferential methylation of that part of DNA which is presumably involved in the control of transcription was thus expected. With this in mind, it might be easier to accept methylation as a probable controlling factor for differentiation [1,2,4,5]. Formation of a 'minor' thymine (as distinct from that of 5-methyl-cytosine which complements guanine as does cytosine itself) would complement adenine and constitute an actual genome modification. Consequently, one would expect this modification to occur at the level of regulatory genes: an increase of AT pairs at the expense of GC pairs with successive cell cycles, while the ratio of 5-methyl-cytosine/cytosine should return always to its previous value after each wave of synthesis/methylation. The fate of 'minor' thymine and the control of its quantity during differentiation is however to be studied.

Besides differentiation, fig. 2 has also some bearing on studies of cell transformation concerning the site of attachment of an oncogenic virus RNA to the host genome. Oncovirus RNA can be bound to host DNA, since the normal cells of several animal species contain large numbers of DNA sequences of unknown function which are homologous to it [28–34]. Therefore, hybrids of animal DNA/virus RNA should be appropriate material in which to investigate the site of attachment of the virus genome to the host genome, if the host DNA is preferentially methylated at the level of the acceptor zone of operon (fig. 2c). The preliminary experiments which involved the system Rous sarcoma virus RNA/methylated DNA from isolated nuclei of rat liver showed the hybrids to contain an appreciable amount of ^{3}H counts from the methylated DNA, as did dRNA in fig. 2b (unpublished data). This is in accord with the fact that some RNA oncoviruses contain 20% A [29]. DNA methylation may become, thus, a methodological approach for studying the mechanism of cell transformation by oncornaviruses. The working hypothesis is that the repetitive sequences of the regulatory zone of operon might represent a large target for the oncovirus RNA input.

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